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Body size variation of and multiple blood feeding by Culiseta melanura (Coquillett) in southeastern Massachusetts.

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BODY SIZE VARIATION OF AND MULTIPLE BLOOD FEEDING BY *CULISETA MELANURA* (COQUILLETT) IN SOUTHEASTERN MASSACHUSETTS

A thesis presented

BY

ROBERT ALLAN ANDERSON

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Entomology

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DEDICATION

To my late cousin, Michelle. Her great courage in the face of much pain inspired me to keep going no matter what.

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My experience as a graduate student at the University of Massachusetts has been enhanced by many people. I would like to thank the members of my thesis committee, Drs. Chih-Ming Yin and John G. Stoffolano Jr. for their valuable contributions and advice to my project. I would also like to thank Dr. Tom Scott and Les Lorenz, University of Maryland, for providing me with the opportunity to work in their lab. I am especially grateful to my advisor, Dr. John Edman for his advice, support and encouragement to follow my own mind in my research. I look forward to many more years of research and collaboration with him.

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I owe a debt of friendship to many people at UMASS for making my life here so enjoyable. Lil and John Edman opened their home to me on many occasions. To the Apiary crew, Dennis, Paula, Sangvorn, Russ and Pat: thanks for being my family away from home.

To my parents, brothers and sisters: thanks for the support and for putting up with endless stories about mosquitoes.

ABSTRACT

BODY SIZE VARIATION OF AND MULTIPLE BLOOD FEEDING BY *CULISETA MELANURA* (COQUILLETT) IN SOUTHEASTERN MASSACHUSETTS

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This research was carried out during 1987 and 1988 to collect biological data about factors affecting the vector potential of *Culiseta melanura* (Coquillett) for Eastern Equine Encephalomyelitis (EEE) virus. Resting boxes were used to collect adult *Cs. melanura* and *Cs. morsitans* from the Hockamock swamp at Raynham, MA. Data on wing length, body mass, parity, engorgement frequencies and multiple blood feeding by these species indicate potentially important aspects of the biology of *Cs. melanura* with regard to transmission of EEE virus in Massachusetts.

Eleven hundred and forty female *Cs. melanura* and 317 *Cs. morsitans* were dissected for parity determination or wing length measurement. There was no significant association between parity (a measure of survival) and wing length (an indicator of body size) for either *Cs. melanura* or *Cs. morsitans*. The mean wing length of *Cs. melanura* decreased from the spring generation to the late

summer generation in 1987 and 1988. The mean wing length of Cs. morsitans did not change significantly during 1987 or 1988. The coefficient of variation of wing length of Cs. melanura and of Cs. morsitans indicate that larval development of these species is probably density independent. Data indicate that body size variation in Cs. melanura and Cs. morsitans is not important to survival.

A technique was developed to use rubidium and cesium as host-blood markers for studying blood feeding behavior of mosquitoes in the wild. Limitations of serology for identifying multiple blood-meals taken by mosquitoes from closely related hosts indicated a need for a new approach. Rubidium could be detected in Aedes aegypti mosquitoes that had fed on rubidium marked chickens up to 72 hr after injection of the rubidium at a dose of 500 mg/kg. It was necessary to administer a dosage of 750 mg/kg of cesium chloride to mark chicken blood for 72 hr. Rubidium and cesium are easily distinguished when present in the same mosquito blood-meal. Rubidium and cesium were detected in 10 of 10 Ae. aegypti which had imbibed blood from two hosts; one marked with rubidium and one marked with cesium.

Culex pipiens quinquefasciatus were allowed to engorge on unrestrained chickens, one marked with rubidium and one marked with cesium, placed in the same cage. Thirty one of 73 mosquitoes fed only on the

rubidium injected chicken and 28 mosquitoes fed only on the cesium injected chicken. Fourteen of 73 mosquitoes fed on both chickens. Rubidium and cesium do not appear to differentially affect ability of mosquitoes to engorge on chickens injected with these alkali metals in the laboratory.

Rubidium and cesium were evaluated as host-blood markers to study multiple blood feeding of Cs. melanura in the Hockamock swamp. Rubidium and cesium can be identified in Cs. melanura that have fed on chickens injected with these alkali metals. Resting boxes are effective for collecting Cs. melanura that have fed on marked chickens in a field situation. One hundred and ninety one Cs. melanura were positive for rubidium or cesium or both out of 721 engorged females collected during 1987 and 1988. A total of 15 engorged females were positve for both rubidium and cesium. Data indicate that this technique is practical for the study of blood feeding behavior and specifically multiple blood feeding on closely related hosts. Information from such studies may contribute to the development of more accurate models of disease transmission.

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CHAPTER I

BODY SIZE VARIATION AND PARITY OF *CULISETA MELANURA* (COQUILLETT) AND *CULISETA MORSITANS* (DYAR) IN SOUTHEASTERN MASSACHUSETTS

Introduction

Recent research (Haramis 1985, Hawley 1985, Landry et al. 1988, Nasci 1986a, 1986b, 1988) has focused on the relationship between adult mosquito body size and vectorial capacity. These studies primarily dealt with container breeding mosquito species. The response of these species to environment and food supply is such that larval habitat limitation (in terms of available nutrients) can result in a great deal of variability in the size of emerging adult mosquitoes (Fish 1985). In those cases where adult survival is correlated with increasing body size, large female mosquitoes may have a higher vectorial capacity than smaller ones (Nasci 1986a, 1986b, 1988). Mosquitoes which live longer have a greater probability of becoming infected with a pathogen and also a greater probability of surviving the extrinsic incubation period of the disease agent long enough to become infective and to transmit it to uninfected hosts. This has implications both for sampling mosquito populations for risk assessment and for mosquito control

methods. The only permanent-water species for which this relationship has been investigated is Culex salinarius (Nasci 1986a).

Cs. melanura is the principle enzootic vector of Eastern Equine Encephalomyelitis (EEE) virus in the United States (Burbutis and Jobbins 1957, Hayes et al. 1962, Rice and Pratt 1972, Morris et al. 1980, Nasci and Edman 1981). It is common in moated, brown-water (acid) forest swamps from New England to central Florida. Females of this species are multivoltine and oviposit in cool permanent pools of water under the roots of large trees. Larvae develop slowly over a period of 2 to 6 weeks (Morris et al. 1976). Mated females readily leave the dense vegetation of the swamp interior to blood feed in peripheral upland areas of human habitation (Joseph and Bickley 1969, Morris et al. 1976, Nasci and Edman 1981). Although there is no evidence of Cs. melanura serving as an epizootic and epidemic vector of EEE virus to horses and humans, its importance as a maintenance and amplification vector of EEE virus among birds is well documented (Edman et al. 1972a, Rice and Pratt 1972, Nasci and Edman 1981).

Cs. morsitans is a univoltine, ornithophilic mosquito species (Nasci and Edman 1981, Morris et al. 1976) which occurs in the same swamp habitat as Cs. melanura. It is a much larger species than Cs. melanura and so it may be subject to different environmental

constraints. Cs. morsitans overwinters as eggs (Wallis and Whitman 1968), whereas Cs. melanura overwinters as larvae (Joseph and Bickley 1969). Ecological differences between Cs. melanura and Cs. morsitans may account for differences in the importance of body size to survival for each species.

Information on parity and body size of Cs. melanura and Cs. morsitans may indicate the relative importance of adult body size to adult survival for these species. Although parity data have been published for Cs. melanura (Morris et al. 1980, Nasci and Edman 1984), they have not been related to body size in this species. The ecology of Cs. melanura is very different from that of container breeding mosquito species such as Aedes triseriatus. The larval developement time of Cs. melanura may be 2 to 6 weeks (Joseph and Bickley 1969), pupation and adult emergence is not synchronous, and the multiple generations blend into each other during late summer in Massachusetts, making identification of cohorts difficult. Fish (1985) suggested that expected variability in body size for this type of mosquito species would be low. Under these circumstances, one would expect body size to be less important in determining survival than for a species with greater body size variation. On the other hand, Cs. melanura is a long-lived species (Joseph and Bickley 1969) and this aspect of its ecology would be expected to place a

premium on life history traits which affect survival. Thus, data on body size and survival are important in clarifying the importance of environmental constraints on this species.

Cs. morsitans provides an opportunity to compare body size and survival data with that of Cs. melanura. Although adults of Cs. morsitans are collected in the same habitat, they overwinter as eggs. This is different from Cs. melanura which overwinters as larvae. Only one generation of Cs. morsitans emerge to the adult stage per year so that identification of a discrete cohort is possible. If differential body size observed for this species is important to survival, one would expect to observe an increase in the proportion of large, parous (old) mosquitoes as the season progresses, especially as recruitment is from a single cohort.

The relationship between adult body size and adult survival was examined for Cs. melanura and Cs. morsitans to relate this to vectorial capacity for EEE virus in southeastern Massachusetts.

Materials and Methods

Research Location

Research sites were chosen in slightly open, upland areas of the Hockamock Swamp near Raynham, Ma (Nasci and Edman 1981). These sites were chosen because they can be sampled easily for Cs. melanura and Cs. morsitans using the resting box described by Edman et al. (1968). Twenty

four resting boxes were placed in various locations, usually at the bases of trees or rock piles so that the openings faced away from the morning sun (Morris et al. 1976). This type of placement kept resting boxes cool and humid in the morning and delayed movement of mosquitoes to more favorable microhabitats. Boxes were placed in groups of 6 in three different locations about 1 mile apart within the Hockamock swamp.

Collection and Preparation

Collections were made weekly from May to August in 1987 and from May to September in 1988. A battery powered aspirator, modified from Edman (1979), was used to collect resting mosquitoes from the resting boxes between 0700 and 0900 hours. Mosquitoes were placed in a Thermos cooler with icepacks, transported to the laboratory and killed at -20 C. Specimens were identified to species, sorted by sex, and visually graded into empty, engorged, or gravid categories. Samples of non-blood-fed Cs. melanura and Cs. morsitans were set aside for dissection to determine parity status (Detinova 1962) and for body size determination by wing-length measurement (Christophers 1960).

Wing length was measured from the axillary incision to the tip of the wing (not including fringe scales) using a dissecting microscope fitted with an ocular micrometer. Measurements were converted to millimetres. A control sample of dried, adult, female Cs. melanura (n

= 67) was weighed and wing length was measured; the relationship between body weight and wing length was determined to be linear over the range of wing lengths of wild-caught females (r^2 = 0.621). Thus, it was considered unnecessary to convert wing length to body weight as Fish (1985) suggested.

Total numbers of males and females were counted. Numbers of males expressed as percentages of total mosquitoes within a species were used in conjunction with peaks in the percent of nulliparous females to establish the duration of generations of Cs. melanura.

Data Analysis

Mean wing length of female Cs. melanura and Cs. morsitans were compared by sampling date (ANOVA). Mean wing lengths of parous and nulliparous mosquitoes by sampling date were also compared using ANOVA (NH Analytical Software). The coefficient of variation of the wing length measurements of Cs. melanura and Cs. morsitans was also determined to provide insight into larval developmental ecology (Fish 1985).

Results

A total of 6117 adult Cs. melanura, including 3435 males and 2682 females were collected from resting boxes in 1987 (Table 1). The data for percentage of males and nulliparous females (Figure 1) indicate that there were 4 distinct generations of Cs. melanura in 1987. Sampling

was not continued through September, so one late generation of females may have been missed. Males emerge about 2 weeks before females (Morris et al. 1976). Wing length was measured for 624 non-blood fed females (Table 2). The coefficient of variation for these wing lengths was 7.49 (Table 6). Three hundred and eighty seven non-blood-fed, non-gravid Cs. melanura females were dissected for parity determination (Table 1). The frequency of parous females was 28% (110/387). The mean wing length of female Cs. melanura decreased significantly (ANOVA $p < 0.0001$) from the beginning of the sampling period to the end (Table 2). The mean wing length of parous females was generally greater, (though not significantly so) than that of nulliparous females except for the beginning of the sampling period (Table 2).

A total of 4311 adults of Cs. melanura, including 2006 males and 2305 females, were collected from resting boxes in 1988 (Table 1). The percentages of males and nulliparous females (Figure 2) indicate 4 generations of Cs. melanura. Wing length and parity were determined for 512 non-blood-fed, non-gravid female Cs. melanura (Table 4). The coefficient of variation of the wing length measurements was 5.69 (Table 6). The percentage of parous mosquitoes was 30% (153/512) (Table 1). The mean wing length of female Cs. melanura was significantly less at the end of the sampling period than at the beginning

(ANOVA $p < 0.0001$) (Table 3). Wing lengths of parous mosquitoes were not consistantly larger than those of nulliparous mosquitoes (Table 3).

A total of 113 adult female Cs. morsitans was collected from resting boxes in 1987 (Table 1). They were not dissected for parity. Wing length did not change significantly from the beginning of the sampling period to the end (Table 4). The coefficient of variation of wing length measurements was 4.55 (Table 6). Three hundred and nineteen adult females of this species were collected from resting boxes in 1988 (Table 1). Fifteen were gravid, 8 were blood-fed and 296 were empty. Of the 201 non-blood-fed/non-gravid females dissected for parity determination, 169 were nulliparous and 32 were parous (Table 1). Wing lengths did not vary significantly over the sampling period (Table 5). The coefficient of variation of the wing length measurements was 4.19 (Table 6). There were no significant differences between the wing lengths of nulliparous and parous mosquitoes (Table 5).

Discussion

The mean wing length of Cs. melanura decreased over the trapping season during both 1987 and 1988. This pattern is consistant with that observed for Aedes triseriatus in Indiana tire dumps (Haramis 1983), Aedes sierrensis collected from treeholes in Oregon (Hawley

1985) and with data for Cs. melanura in Maryland (Lorenz personal communication). This general decrease in the average wing length may be a result of accelerated larval development caused by increased temperature as the season progresses. Although larval crowding has been suggested as a factor which reduces available food (Fish 1985), Cs. melanura is seldom found at high larval densities (Joseph and Bickley 1969). Nasci (1988) observed an increase with respect to season in the mean wing length of Aedes triseriatus females collected from shaded tires in Louisiana and he speculated that this was due to a decrease in larval density and a concommitant reduction in larval competition for nutrients. Cooler temperatures may also have increased larval development time sufficiently to account for the increase in size. Lorenz (personal communication) observed a late-season increase in the mean wing length of Cs. melanura females collected from the Pokomoke swamp in Maryland. Wing length measurements did not return to early season values, but the increase was significant. This reverse in wing length trend was probably due to decreasing water temperature which resulted in longer development time. Cs. melanura can be collected for about one month longer in Maryland than in Massachusetts. This may explain the emergence of larger mosquitoes late in the season in Maryland but not in Massachusetts.

The percentage of parous Cs. melanura females during 1987 and 1988 were similar and combined with the prevalence of males suggest that there are 4 generations of Cs. melanura in Massachusetts. These data agree with those of Nasci and Edman (1984) who suggested 4 generations on the basis of collections from 2 resting boxes placed in the Pine swamp in Massachusetts in 1978. The fourth generation of mosquitoes emerges during September and adults can be collected until late October in the absence of severe frosts. However, low population levels combined with low night-time temperatures may inhibit host-seeking activity and may reduce the contribution of the fourth generation to the pool of overwintering larvae. The extent of this contribution remains to be investigated and may be significant in years when a mild fall allows for substantial mosquito survival, blood feeding and subsequent oviposition activity.

The absence of any significant difference between the wing length of parous and nulliparous Cs. melanura and the absence of a consistant year to year trend in the wing length of parous and nulliparous females relative to each other (Table 2, Table 4) indicate that body size is probably not an important determinant of survival for Cs. melanura. Analysis of the data by comparison of mean wing length for old (parous) and young (nulliparous) female mosquitoes failed to indicate a significant

relationship (Table 2, Table 4). It is instructive to consider the coefficient of variation (CV) for the wing length of Cs. melanura (Table 6) which is equal to 6.59. This is less than half the CV of Ae. triseriatus, a species for which a relationship between survival (parity) and body size (wing length) has been demonstrated (Nasci 1988, Haramis 1983, 1985). Of significance is the fact that body size of Aedes triseriatus is highly variable (Fish 1985). It is likely that the total variation in body size of Cs. melanura is insignificant in terms of survival. This question remains to be investigated as no laboratory data are available to link differential survival of adult Cs. melanura directly with a given percent variation in body size. Of course, analysis of this relationship may be complicated by the asynchronous emergence of Cs. melanura broods, as well as the overlapping generations. Continuous recruitment of newly emerged females into an aging population may tend to obscure differential survival or blood feeding of small and large mosquitoes.

Data for Cs. morsitans provide a useful comparison with those for Cs. melanura. Culiseta morsitans is univoltine (Wood et al. 1979) and is a significantly larger mosquito species than Cs. melanura (Tables 2, 3, 4, 5). Although there is only one generation per year (Morris et al. 1976, Wallis and Whitman 1968), the emergence of adult Cs. morsitans is very asynchronous.

Adult emergence occurs over a period of about 1 month. Culiseta morsitans was included in this study because the single generation could be sampled accurately. Based on the hypothesis that larger sized mosquitoes survive longer, it was expected that there would be an accumulation of large, parous mosquitoes by the end of the Cs. morsitans season. There was an increase in the percentage of parous mosquitoes, although not to the percentage observed by Morris et al. (1976), but the wing length of parous mosquitoes was not significantly different from that of nulliparous mosquitoes (Table 5). These data suggest a similar conclusion for Cs. morsitans as for Cs. melanura, i.e. body size does not exert a significant survival effect. One must also keep in mind that the coefficient of variation of wing length (Table 6) for this species is very low --- a similar situation to Cs. melanura. It is likely that the low variability in body size translates into an insignificant biological difference between the "small" and "large" size extremes.

Data presented here do not support the hypothesis that larger adult body size is important to adult survival for Cs. melanura and Cs. morsitans. It is clear that conclusions can not be drawn across species lines. The importance of adult body size varies from species to species according to life history parameters (Nasci 1986a, 1988, Hawley 1985, Haramis 1983, 1985) and even within a species depending on geographical location

(Landry et al. 1988, Nasci 1988). The ecology of a species, as well as genetic potential for size variation are important factors which ultimately may have an impact on vectorial capacity.

Culiseta melanura overwinter as larvae and Cs. morsitans overwinter as eggs (Wallis and Whitman 1968). These are significant departures from the pattern of adult overwintering observed for most other Culiseta species (Wood et al. 1979). It is perhaps more logical to examine those species which overwinter as adults to determine if large adult body size confers a significant advantage for survival during the more adverse environmental conditions of winter.

Data presented in this study do not support the hypothesis that large adult Cs. melanura or large Cs. morsitans live longer than do small adults of these species. Based on the hypothesized relationship between longevity and vector potential, I can not conclude that large females of Cs. melanura and Cs. morsitans are better vectors than small females of these species. However, body size may be important to vector potential in terms of ability to transmitt virus (Grimstad and Haramis 1984). Such information is important in helping to explain why some mosquito species are disease vectors and others are not.

Table 1. Numbers of Culiseta melanura and Culiseta morsitans collected from resting boxes in Raynham, MA, during 1987 and 1988.

	Culiseta melanura		Culiseta morsitans	
	1987 #(%)	1988 #(%)	1987 #(%)	1988 #(%)
Nullipars	287 (72)	359 (70)	--	169 (84)
Pars	110 (28)	153 (30)	--	32 (16)
Examined	397(100)	512(100)	0	201(100)
Unexamined	2285	1793	113	118
Total ♀	2682 (44)	2305 (53)	113(100)	319(100)
Total ♂	3435 (56)	2006 (47)	--	--
Total ♂ and ♀	6117(100)	4311(100)	113(100)	319(100)

Table 2. Size variation by sampling date of Culiseta melanura collected in Raynham, MA, during 1987.

	Nullipars		Pars		Combined[1]	
	Wing length (mm)					
Date[2]	$\bar{X}$ (SD)	n	$\bar{X}$ (SD)	n	$\bar{X}$ (SD)	n
154	--		--		4.22(0.17)	17[3]
165	4.16(0.21)	65	4.20(0.24)	16	4.17(0.22)	94
173	4.13(0.26)	25	4.05(0.24)	7	4.04(0.25)	64
175	4.06(0.20)	29	4.05(0.17)	9	4.06(0.19)	40
183	3.84(0.20)	28	4.13(0.16)	6	3.89(0.21)	37
189	3.94(0.18)	24	4.13(0.20)	14	4.01(0.21)	38
197	3.87(0.20)	26	4.01(0.21)	14	3.92(0.21)	40
204	3.91(0.22)	29	3.96(0.18)	14	3.90(0.23)	49
211	3.72(0.25)	30	3.93(0.13)	13	3.77(0.24)	45
217	--		--		3.73(0.21)	64[3]
226	3.65(0.26)	21	3.82(0.28)	17	3.74(0.28)	40
231	--		--		3.74(0.30)	69[3]
239	--		--		3.61(0.23)	27[3]
Overall	3.96(0.28)	277	4.02(0.24)	110	3.92(0.29)	624

1 Samples may include some individuals for which wing length, but not parity was determined.
2 Based on the sequential Julian calendar.
3 Samples were not dissected.

Table 3. Size variation by sampling date of Cullseta melanura collected in Raynham, MA, during 1988.

	nulIpars		pars		combined[1]	
	Wing length (mm)					
Date[2]	$\bar{X}$ (SD)	n	$\bar{X}$ (SD)	n	$\bar{X}$ (SD)	n
146	4.38(0.18)	18		0	4.38(0.18)	18
162	4.19(0.26)	26	4.19(0.16)	10	4.19(0.24)	36
167	4.08(0.21)	12	4.22(0.19)	15	4.16(0.21)	27
176	4.04(0.20)	17	4.27(0.22)	12	4.14(0.23)	29
180	4.01(0.10)	8	4.04(0.15)	11	4.03(0.13)	19
188	4.06(0.18)	22	4.10(0.17)	6	4.07(0.18)	28
195	4.07(0.27)	16	4.11(0.18)	7	4.08(0.24)	23
203	3.95(0.22)	32	3.76 --	2	3.94(0.22)	34
209	3.88(0.22)	16	4.07(0.24)	22	3.99(0.24)	38
216	3.87(0.31)	4	3.92(0.29)	13	3.91(0.28)	17
223	3.74(0.16)	23	3.84(0.26)	14	3.78(0.20)	39
229	3.73(0.16)	26	3.80(0.11)	10	3.75(0.15)	37
236	3.79(0.17)	27	3.64(0.17)	5	3.77(0.18)	32
242	3.73(0.22)	33	3.89(0.07)	2	3.74(0.22)	35
250	3.76(0.20)	19	3.87(0.22)	5	3.78(0.20)	24
255	3.80(0.31)	29	3.80(0.21)	10	3.82(0.32)	40
264	3.70(0.15)	14	3.65(0.20)	5	3.68(0.16)	19
273	3.84(0.33)	17	3.63(0.14)	4	3.80(0.31)	21
Overall	3.91(0.28)	359	3.99(0.27)	153	3.93(0.28)	516

1 These samples may have some individuals for which wing length but not parity was determined.
2 Based on the sequential Julian calendar.

Table 4. Size variation by sampling date of *Culiseta morsitans* collected in Raynham, MA, during 1987[1].

Date[2]	Wing length (mm) $\bar{X}$	(SD)	n
165	5.52	(0.27)	18
173	5.49	(0.20)	11
175	5.36	--	2
183	5.50	(0.20)	11
189	5.57	(0.23)	23
197	5.41	(0.25)	16
204	5.62	(0.12)	9
218	5.50	(0.27)	16
226[3]	5.42	(0.40)	7
Overall	5.51	(0.25)	113

1 No parity determinations were made in 1987.
2 Based on the sequential Julian calendar.
3 Some mosquitoes from this sample were actually collected later than this, but were combined because of small sample size.

Table 5. Size variation by sampling date of *Culiseta morsitans* collected in Raynham, MA, during 1988.

	Nullipars		Pars		Combined[1]	
	Wing length (mm)					
Date[2]	$\bar{X}$ (SD)	n	$\bar{X}$ (SD)	n	$\bar{X}$ (SD)	n
162	5.50(0.23)	18		0	5.50(0.23)	18
167	5.45(0.30)	26	5.84(0.00)	1	5.46(0.29)	28
176	5.51(0.19)	23	5.64(0.14)	6	5.54(0.18)	30
180	5.50(0.21)	18	5.39(0.18)	3	5.48(0.21)	21
188	5.58(0.19)	11	5.62(0.17)	6	5.60(0.17)	18
195	5.54(0.28)	30	5.60(0.18)	5	5.57(0.25)	41
203	5.49(0.24)	25	5.37(0.33)	6	5.47(0.26)	31
209	5.55(0.17)	14	5.56(0.17)	2	5.57(0.16)	19
216[3]	5.60(0.27)	4	5.49(0.18)	3	5.54(0.21)	8
Overall	5.51(0.24)	169	5.54(0.22)	32	5.52(0.23)	214

1 These samples may have mosquitoes for which wing length but not parity was determined.
2 Based on the sequential Julian calendar.
3 This sample contains several mosquitoes collected at later dates and combined because of small sample sizes.

Table 6. Coefficient of variation (CV) of wing length of Culiseta melanura and Culiseta morsitans collected in Raynham, MA, during 1987 and 1988.

Year	Culiseta melanura CV(n)	Culiseta morsitans CV(n)
1987	7.49(821)	4.55(113)
1988	5.69(512)	4.19(214)
Mean value	6.59	4.37

Figure 1. Percentage of male and nulliparous female Cullseta melanura collected in Raynham, MA, during 1987

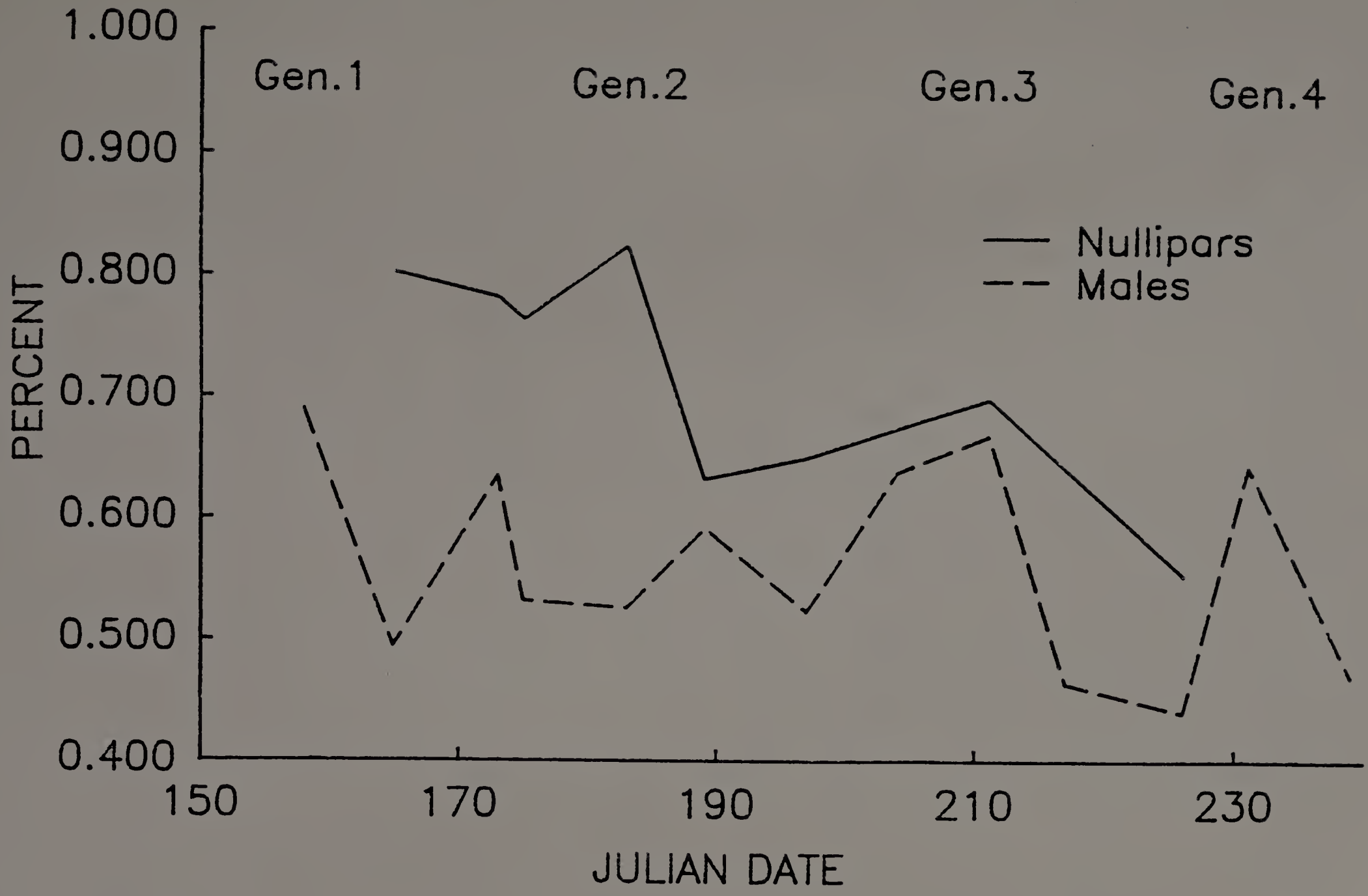
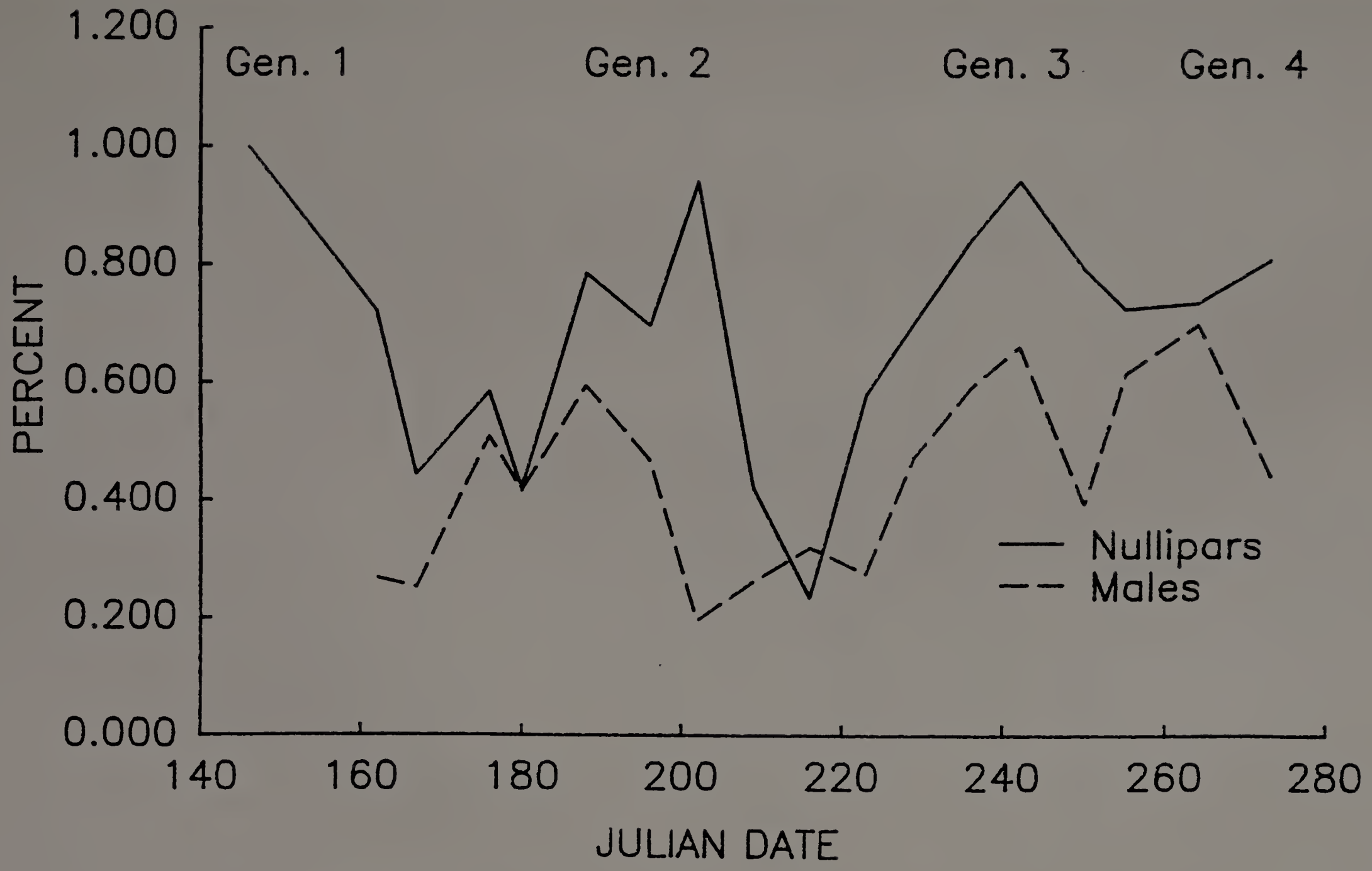

1.000
0.900
0.800
0.700
0.600
0.500
0.400
PERCENT
Gen.1
Gen.2
Gen.3
Gen.4
Nullipars
Males
150
170
190
210
230
JULIAN DATE

Figure 2. Percentage of male and nulliparous female *Cullseta melanura* collected in Raynham, MA, during 1988


Gen. 1
Gen. 2
Gen. 3
Gen. 4
1.200
1.000
0.800
0.600
0.400
0.200
0.000
PERCENT
140
160
180
200
220
240
260
280
JULIAN DATE
Nullipars
Males

CHAPTER II

USE OF RUBIDIUM AND CESIUM AS HOST-BLOOD MARKERS TO STUDY MULTIPLE BLOOD FEEDING OF CULISETA MELANURA IN THE FIELD

Introduction

Models which describe the epidemiology of arthropod borne diseases generally account for only 1 host contact per gonotrophic cycle (Macdonald 1952, de Moor and Steffens 1970, Nasci 1980, Scott et al. 1983). There is ample evidence from the literature to support the contention that many mosquito species take multiple, small bloodmeals during one gonotrophic cycle (Edman and Downe 1964, Edman et al. 1975, Boreham 1975, Klowden and Lea 1979, Mitchell et al. 1979, Nasci and Edman 1981, Washino and Tempelis 1983). However, the evidence is inconclusive as to the importance of multiple feeding in disease transmission (Boreham 1975). Estimates of multiple blood-feeding vary from one species to another (Washino and Tempelis 1983) and also may vary within a species depending on time of year, type of host fed on and mosquito biting pressure (Nasci and Edman 1981).

Multiple probing and blood-feeding events are the result of unsuccessful blood location attempts (Ribeiro 1987) or interruption of actual blood feeding (Edman and Kale 1971, Kale et al. 1972, Walker and Edman 1986) followed by resumption of probing and blood feeding. Unsuccessful probing can result from deficiencies in

salivary enzymes (Ribeiro 1987) or selection by the arthropod of feeding sites with few capillaries (Walker and Edman 1985a). Incomplete blood feeding may result from host defensive behavior (Klowden and Lea 1979, Kale et al. 1972). Resumption of blood feeding appears to be a function of blood-meal size (Klowden and Lea 1978) and energy reserves (Walker and Edman 1985b) although other important factors also may be involved. The cumulative result of multiple probing and multiple blood feeding is potentially higher vectorial capacity associated with multiple chances of acquiring pathogen infection and multiple chances of transmitting the pathogen in each gonotrophic cycle (DeFoliart et al. 1987, Boreham 1975).

Measurement of multiple blood feeding by mosquitoes has been limited to serologically identifying mixed meals from distantly related heterospecific hosts (Washino and Tempelis 1983). Limitation is largely due to cross-reactivity of antigens present in animals of close phylogenetic relationship (Washino and Tempelis 1983). Advances in immunological techniques allow more precise identification of arthropod blood-meals (Washino and Tempelis 1983), but absorption of cross-reacting antibodies, necessary for more specific host identification, is laborious and expensive. Studies of host choice by hematophagous arthropods in the field may be hampered by lack of refrigeration for preservation of blood-fed insects and antisera (Kimsey and Kimsey 1984).

Quantitative information on multiple blood feeding by infected and uninfected vectors would allow more precise estimation of vectorial capacity of these species (Boreham 1975).

An alternative approach is to mark the blood of potential hosts of mosquitoes such that the marker can be identified in the engorged mosqutioes by techniques other than serology. Rubidium is an alkali metal that has been used successfully to mark phytophagous insects for dispersal studies (Berry et al. 1972, Stimmann 1974, Van Steenwyk et al. 1978). Kimsey and Kimsey (1984) demonstrated in a preliminary study that rubidium could be injected into vertebrates and then detected in mosquitoes which had fed on those animals. Cesium has also been used to mark pink bollworm (Moss and Van Steenwyk 1982). Both rubidium and cesium can be identified by atomic emission flame spectrophotometry (Kimsey and Kimsey 1984, Moss and Van Steenwyk 1982) and can be easily distingushed from each other based on different emmission wavelengths. Their data suggested that the blood from more than one bait host can be reliably identified in a blood-fed mosquito by providing at least two hosts, each marked with a different metal.

Multiple blood feeding by Cs. melanura (Coquillett) is of particular interest because this species is the primary enzootic vector of Eastern Equine Encephalomyelitis (EEE) virus to birds throughout the

eastern United States (Burbutis and Jobbins 1957, Hayes et al. 1962, Edman and Kale 1972a, Morris et al. 1980, Nasci and Edman 1981). Nasci (1980) models the 1973 outbreak of EEE virus activity in Massachusetts. The number of adult Cs. melanura predicted by this model exceed population estimates based on larval density, available habitat and survival. This may have been due to other mosquito species which contribute to the spread of the disease (Crans and Schulze 1986, Crans et al. 1986) or because the vectorial capacity of Cs. melanura is actually higher than estimated. Multiple blood feeding by this species on heterospecific hosts ranges as high as 40% (Nasci and Edman 1981). If multiple blood feeding on conspecific hosts is comparable, then vectorial capacity may be greatly underestimated. The additional host contacts which result from multiple feeding provide additional opportunity for the infected mosquito to transmit disease.

The objectives of the present study were: 1) to evaluate in the laboratory the reliability of rubidium and cesium as host-blood markers, 2) to demonstrate that rubidium and cesium can be used in a field situation to monitor and study multiple blood feeding by wild mosquitoes. Aedes aegypti (Linnaeus) and Culex pipiens quinquefasciatus Say were chosen for the initial laboratory studies because they feed more readily under artificial conditions. The importance of Cs. melanura

has already been established and it was felt that further information on the multiple blood feeding habits of this species in the wild was necessary to more accurately estimate its vector potential. It is expected that this technique will be useful to study many diseases transmitted by wild mosquitoes.

Materials and Methods

The feasibility of using rubidium and cesium as easily distinguishable blood markers was evaluated by injecting intraperitoneally 5 week old brown wheaton or white leghorn chickens with 500 mg/kg isotonic rubidium chloride (18.62 mg/ml) or cesium chloride (25.97 mg/ml) in sterile reagent grade water (Kimsey and Kimsey 1984). Two chickens were injected with rubidium and two with cesium. Two other chickens were injected with comparable volumes of sterile, double distilled, deionized water as negative controls. Sugar starved Ae. aegypti mosquitoes (Rockefeller strain) were allowed to feed on the chickens 24, 48 and 72 hr after injection. Mosquitoes were held for 24 hours on 3% sucrose before being killed by freezing.

Laboratory Evaluations

A minimum of two fully engorged mosquitoes from each of the control and experimental chickens for each feeding time were wet ashed according to the method described by Kimsey and Kimsey (1984). The clear, yellowish solutions

were diluted to 3.0 ml with 200 ppm (final concentration) potassium chloride (KCl) in double distilled, deionized water. The resulting solutions were allowed to stand at room temperature for 72 hr to allow coalescence of unhydrolyzed lipids. The clear solutions were pipetted to clean tubes and assayed by atomic emission flame spectrophotometry (Kimsey and Kimsey 1984) at 780 nm and 852 nm for the presence of rubidium and cesium respectively. Experimental mosquitoes with concentrations of rubidium or cesium greater than the mean plus 2 standard deviations of the background concentrations of rubidium and cesium in negative control mosquitoes were considered to be marked.

Reliability of this technique for correctly identifying multiple blood meals was examined in the following manner. Twenty Ae. aegypti mosquitoes were allowed to feed one at a time on a rubidium marked chicken until engorgement was approximately half completed. Engorgement was interrupted and the same mosquitoes each time were allowed to continue blood feeding to repletion on a cesium injected chicken. Thirteen of the mosquitoes at least probed a second time and 10 visibly imbibed more blood. The thirteen mosquitoes which had probed a second time were assayed for the presence of both rubidium and cesium.

Injection of large volumes of rubidium or cesium solutions into the peritoneum of chickens could potentially alter bird behavior and consequently affect blood feeding success. Although Kimsey and Kimsey (1984) did not observe a difference in engorgement frequency of Culex tarsalis fed on injected and uninjected quail, there are no data to indicate if cesium acts in the same manner. To answer this question, 25 Cx. quinquefasciatus per night for 10 nights were allowed to feed on two unrestrained chickens, one marked with cesium and one marked with rubidium. The amount of cesium was increased to 750 mg/kg. Four groups of two chickens each were used over the course of this experiment to account for individual variability in susceptibility to mosquito blood feeding (Kale et al. 1972). Chickens #79 and #68 were used during trial 1. Chickens #79 and #64 were used during trial 2. Chickens #79 and #68 were used again during trials 3, 4 and 5 on consecutive nights. Chickens #302 and #396 were used during trials 6 and 7 on consecutive nights and again during trials 8, 9 and 10 on consecutive nights.

Chickens were placed in 0.027 m^3 hardware cloth cages (13 mm mesh) with water and food ad lib. These cages were placed inside mosquito observation cages described by Walker and Edman (1985b) at 1800 hr each evening. Data indicate that most mosquitoes are eaten by chickens if left overnight without a refugia (Day and

Edman 1984). The holding cages served to limit access by the chickens to the bottom half of the observation cages so that mosquitoes could rest safely after blood feeding. Chickens were given 2 hr to settle down before the mosquitoes were released into the observation cage at 2000 hr. Chickens were observed during this period for obvious behavioral differences such as restlessness or depression. Mosquitoes were left in the observation cages until 0600 hr the next morning. They were collected, frozen and scored for engorgement success and blood-meal size. Fully or partially engorged mosquitoes were assayed for the presence of rubidium and cesium.

Field Evaluations

Rubidium and cesium as host-blood markers were also evaluated in the field during 1987 and 1988. Cs. melanura was chosen as the mosquito species of interest because of its role as a vector of EEE virus. Chickens were marked with rubidium or cesium as described. Data obtained from the experiment with Cx. quinquefasciatus indicated that cesium injected at 750 mg/kg results in a reliable mark for three days after injection. Equal numbers of chickens were injected with rubidium or cesium and paired in 0.027 m^3 hardware cloth (13 mm mesh) cages. Twelve chickens (6 injected with each alkali metal) were used together in a group on Julian dates 202 and 203, 1987. A group of six chickens and a group of two chickens were placed at separate experimental sites on

Julian dates 215 and 216, 1987. Two chickens per experimental site were used throughout 1988. Two experimental sites were set up during 1988. Data for consecutive collection nights were combined.

Cages with chickens were placed on the ground at 1600 hr in the Hockamock Swamp, Raynham, MA, in areas where Cs. melanura are readily collected. Cages were surrounded with a circle of resting boxes placed 5 ft away to attract engorged Cs. melanura.

Cs. melanura do not readily enter baffle-type bait traps (Edman, pers. commun.); blood-engorged females of this species are readily collected in resting boxes (Nasci and Edman 1981). Comparison of these two trapping methods for one night in 1987 resulted in no Cs. melanura recovered from a baffle trap baited with two marked chickens compared to 154 Cs. melanura collected from 6 resting boxes surrounding two marked chickens. It was decided to use only resting boxes as a means of recovering female mosquitoes for rubidium and cesium assay.

Mosquitoes were collected from resting boxes each morning before 0900 hr. Mosquitoes were transported in coolers with ice packs to the laboratory where they were killed by freezing. Collections were identified to species, and the number of each sex counted. Engorged Cs. melanura were separated for alkali metal determination.

Mosquitoes were prepared and assayed for rubidium and cesium as described. Resting boxes also were placed 1 mile away in the swamp to monitor engorgement in the absence of introduced hosts. Blood-engorged mosquitoes from this control site were also assayed for the presence of rubidium and cesium to establish background concentrations.

Results

Laboratory Evaluations

Twenty six Ae. aegypti were assayed to determine if both rubidium and cesium effectively mark host blood for 3 days (Table 7). Thirteen of 13 mosquitoes which had fed on the rubidium injected chickens at 24, 48 or 72 hr after injection were marked. Nine of 9 mosquitoes fed at 24 and 48 hr post injection on the cesium injected chickens were marked. Two of 4 fed on the cesium injected chickens 72 hr after injection were unmarked (Table 7). A small amount of rubidium (5 ppb) was detected in one of the mosquitoes fed on a cesium injected chicken, but this reading occurred at a time when the spectrophotometer was fluctuating possibly due to unusually high humidity. The resulting concentration was only slightly above the mean plus 2 standard deviation cutoff value for rubidium and represents an artifact. Rubidium concentrations for all the known positives were much higher than the cutoff value. No false cesium positives were detected.

Thirteen Ae. aegypti at least probed on a cesium injected chicken after blood feeding on a rubidium injected chicken was interrupted. Ten of ten mosquitoes which were observed to imbibe blood a second time were positive for both rubidium and cesium. The three mosquitoes for which no visible engorgement occurred during refeeding were only positive for rubidium.

Over the course of ten nights, 73 blood-engorged Cx. quinquefasciatus were recovered from the cage with the paired chickens (Table 8). The percentage of engorged mosquitoes varied from night to night, as did the number of mosquitoes eaten by the chickens (Table 8). Rubidium or cesium or both were detected in all engorged mosquitoes which were recovered. The cesium signal was strong in all the mosquitoes, including those which engorged 72 hr after the chickens were injected. Thirty one of the blood-engorged mosquitoes were positive for rubidium and 28 were positive for cesium. Fourteen were positive for both rubidium and cesium. Multiple blood feeding also varied from night to night (Table 8).

Field Evaluations

Total numbers of female Cs. melanura and numbers of blood-engorged females collected during 1987 and 1988 at the experimental and control sites for each sampling date are given in Table 9. The percentage of engorged mosquitoes at the experimental sites with the marked chickens was comparable to that at the control site in

1987. The percentage of engorged mosquitoes at the experimental sites was greater than at the control site in 1988 (Table 9). Engorgement frequencies fluctuated a great deal from night to night and from year to year.

Numbers of unmarked, and rubidium-marked and cesium-marked engorged females collected from the experimental sites during 1987 and 1988 are given in Table 10. Data from consecutive nights are combined.

One hundred and one engorged mosquitoes were collected in 1987. Thirty four mosquitoes were positive for rubidium and 16 were positive for cesium. Five of the mosquitoes collected on Julian date 203 were positive for both rubidium and cesium. None of the engorged mosquitoes, collected from the site with only 2 chickens on Julian dates 216 or 217, were marked. The percentage of blood-engorged Cs. melanura which were marked varied from a low of 35 to a high of 61%.

The experimental sites yielded 620 Cs. melanura during 1988; 136 were marked --- 95 with rubidium and 31 with cesium (Table 10). Ten females were positive for both rubidium and cesium; five collected on Julian date 167, two on Julian date 168, and one each on Julian dates 176, 203 and 209. The percentage of blood-engorged Cs. melanura which were marked varied from < 1% to 100% (Table 10).

Discussion

Preliminary data (Table 7) indicated that rubidium and cesium could be used together as a marking system and could be easily distinguished from each other using atomic emission flame spectrophotometry. Observations of chicken behavior after injection with either rubidium or cesium indicated no long term behavioral changes that might affect mosquito blood feeding success. Transient (< 1 hr) depression was observed for some of the birds and this is attributed to the injection of fluid into the peritoneum rather than a toxic effect of the alkali metals (Kimsey and Kimsey 1984).

The one mosquito which was falsely positive for rubidium (Table 7) may cause mild concern about the accuracy of the technique. However, the fluctuating behavior of the spectrophotometer at the time of this reading is the likely cause of this artifact. It became standard procedure to always read control solutions during the entire assay period to correct for drift in the baseline values set by the standard solutions. This precaution allowed the detection of relatively low levels of rubidium or cesium in poorly marked mosquitoes. Mosquitoes were well marked in over 90% of the samples processed. Data also indicate that the duration of a mark with cesium is less than that of rubidium when both are injected at identical dosages. When the dosage of

cesium was increased to 750 mg/kg, the detectability, from mosquito samples, of the mark was extended to 3 days.

Known double blood-meals were also readily identified. The 3 mosquitoes observed to probe a second time without visible blood intake were negative for cesium, but positive for rubidium. These data indicate that either no blood was imbibed from the cesium injected chicken or that the amount of blood was insufficient for detection of cesium. An approach that could be used to determine the smallest amount of blood from an injected host which will result in a detectable signal is to administer varying amounts of marked blood by enema. This would help to standardize the technique more precisely.

Taken collectively, these data suggest that mosquitoes allowed to blood feed on unrestrained hosts do not preferentially feed on rubidium marked versus cesium marked individuals (Table 8). Ratios varied greatly from night to night. Rubidium marks dominated the blood-fed mosquitoes collected some nights and cesium dominated the marks other nights. Night to night bias in rubidium marked or cesium marked mosquitoes may result from individual differences in susceptibility of hosts to mosquito attack (Kale et al. 1972). This may be an important consideration to programs which monitor blood

feeding patterns of mosquitoes and underscores the necessity of using many different individual birds as hosts.

Data indicate that the addition of domestic chickens to a wild habitat may influence engorgement of wild Cs. melanura (Table 9). No consistant pattern was observed but engorgement was higher at the experimental sites than at control sites for many of the nights when marked chickens were used. The obvious explanation is that the introduction of additional blood sources in the form of chickens increases the blood feeding opportunities for mosquitoes. Food provided for the caged chickens also may have attracted wild birds to the vicinity of the resting boxes. The importance of aggregations of birds to mosquito engorgement, especially during periods of virus transmission, should be investigated further (Edman et al. 1972b).

The initial objective in evaluating the rubidium and cesium host-blood marking technique in the field was to establish that sufficient marked blood-meals could be recovered to make the technique useful. The percentage of engorged Cs. melanura which were marked in 1987 and 1988 varied from less than 1% to 100%. Although many blood-fed mosquitoes were recovered on Julian dates 202, 203, and 204 1988, only one was marked (Table 10). It rained almost 24 hr per day that week and the chickens were not protected from rainfall. Stress created by the

weather may have prevented blood feeding by Cs. melanura. The large number of engorged mosquitoes might have fed on wild, unmarked birds. In general, there were sufficient marked mosquitoes recovered to indicate that this technique is useful for studying blood feeding behavior.

The second objective relating to the use of rubidium and cesium as host-blood markers for Cs. melanura was to gather preliminary data on multiple blood feeding by this species on conspecific hosts. Data indicate that multiple blood feeding on conspecific hosts does occur in nature. It appears to be highly variable but often related to periods when large numbers of mosquitoes engorge (Table 10). Edman et al. (1972b) demonstrated that increased mosquito densities often resulted in increased numbers of incomplete blood-meals. Data on multiple blood feeding (Table 10) agree with the notion that increased numbers of incomplete blood-meals will be observed on nights when mosquito densities are high. If one accepts the hypothesis that conditions which are conducive to interrupted blood feeding are also conducive to multiple blood feeding, the dynamics of multiple blood feeding may be influenced by an interplay of such factors as host density, host species, host behavior and infection with a disease organism in the host or mosquito (Edman and Kale 1971, Day and Edman 1983, Walker and Edman 1985a, 1986, Edman and Scott 1987). Investigations

are continuing into the relative importance of these factors to multiple blood feeding and disease transmission.

Data indicate that host-blood marking using the alkali metals rubidium and cesium is a feasible method to study some aspects of mosquito / host interactions. It is especially applicable to the problem of quantitating multiple blood-meals taken from serologically indistinguishable hosts --- a factor which may be of prime epidemiological importance to disease transmission systems that are host and vector specific. Information derived from studies of the factors involved in multiple blood feeding will contribute to more accurate biological data used to model and predict disease outbreaks. This is predicated on the assumption that infected mosquitoes can transmit a disease organism more than once. This assumption has been confirmed experimentally by Grimstad et al. (1980) for LaCrosse virus and Ae. triseriatus. This technique may also prove to be useful for answering other fundamental questions related to blood feeding behavior of hematophagous arthropods.

Table 7. Numbers of rubidium and cesium containing *Aedes aegypti* mosquitoes that previously had fed on chickens injected with 500 mg/kg rubidium or 500 mg/kg cesium.

Chick no. (metal)	Time post injection (hours) 24	48	72	Total
	no. marked / no. fed			
1 (RB)	3/3	2/2	2/2	7/7
2 (RB)	2/2	2/2	2/2	6/6
3 (CS)	3/3	2/2	1/2	6/7
4 (CS)	2/2[1]	2/2	1/2	5/6

1 One of these mosquitoes was weakly positive for rubidium (see text for explanation).

Table 8. Blood feeding success of *Culex quinquefasciatus* exposed to paired, unrestrained chickens marked with rubidium or cesium.

	Trial no.[1] 1	2	3	4	5	6	7	8	9	10	Total
Mosquitoes	25	25	25	25	25	25	25	25	25	25	250
Eaten	12	10	8	10	10	19	15	14	7	16	121
Unengorged	2	2	8	4	5	5	6	5	13	6	56
Engorged	11	13	9	11	10	1	4	6	5	3	73
Marked	11	13	9	11	10	1	4	6	5	3	73
RB	2	8	3	7	4	0	3	1	1	2	31
CS	9	3	2	2	2	1	1	5	2	1	28
Both	0	2	4	2	4	0	0	0	2	0	14

1 See text for explanation of chickens used for specific trials.

Table 9. Percentage of blood-fed Culiseta melanura collected from resting boxes in the Hockamock swamp, Raynham, MA, during 1987 and 1988.

Year Date[3]	Control[1] Blood-fed n	(%)	Total n	Experimental[2] Blood-fed n	(%)	Total n
1987						
203	25	(15)	168	75	(13)	567
216	13	(12)	114	26	(7)	348
Total	38	(14)	282	101	(11)	915
1988						
163	3	(8)	39	11	(11)	98
168	13	(8)	173	268	(27)	985
176	34	(10)	343	70	(19)	288
196	22	(5)	463	22	(7)	310
203	39	(10)	388	127	(25)	502
209	34	(20)	173	111	(42)	264
242	6	(9)	69	8	(9)	87
250	1	(4)	25	4	(44)	9
255	3	(11)	28	3	(13)	23
Total	155	(9)	1701	624	(24)	2566

1 These resting boxes were placed 1 mile away from the marked chickens.
2 These resting boxes surrounded the cages of marked chickens.
3 Based on the sequential Julian calendar; Appendix I.

Table 10. Percentage of rubidium and cesium marked Culiseta melanura collected from experimental resting boxes in the Hockamock swamp, Raynham, MA, during 1987 and 1988.

Year Date[2]	Total[1] n	Negative n (%3)	Positive n (%3)	RB n (%4)	CS n (%4)	Both n (%4)
1987						
203	75	29 (39)	46 (61)	30 (65)	11 (24)	5 (11)
216	26	17 (65)	9 (35)	4 (44)	5 (56)	0 --
Total	101	46 (46)	55 (54)	34 (62)	16 (29)	5 (9)
1988						
163	10	9 (90)	1 (10)	0 --	1(100)	0 --
168	265	171 (65)	94 (35)	70 (74)	17 (18)	7 (8)
176	70	57 (81)	13 (19)	9 (69)	3 (23)	1 (8)
196	22	16 (73)	6 (27)	2 (33)	4 (67)	0 --
203	127	126 (99)	1 (1)	0 --	0 --	1(100)
209	111	97 (87)	14 (13)	9 (64)	4 (29)	1 (7)
242	8	5 (62)	3 (38)	1 (33)	2 (67)	0 --
250	4	3 (75)	1 (25)	1(100)	0 --	0 --
255	3	0 --	3(100)	3(100)	0 --	0 --
Total	620	484 (78)	136 (22)	95 (70)	31 (23)	10 (7)

1 Total number of blood-fed mosquitoes.
2 Based on the sequential Julian calendar; Appendix I.
3 Percent of total blood-fed mosquitoes.
4 Percent of total marked mosquitoes.

CHAPTER III

CONCLUSIONS

The main conclusions to be drawn from the present study are as follows:

1) There appears to be no relationship between wing length (a measure of body size) and parity (a measure of age) of Cs. melanura or Cs. morsitans. It is unlikely that body size variation in these species exerts any impact on vector potential based on the premise that factors which increase the probability of survival also enhance vector potential.

2) Rubidium and cesium can reliably be used as host-blood markers to study blood-feeding behavior and specifically multiple feeding behavior in the laboratory and in the field.

3) Culiseta melanura engages in multiple feeding on conspecific hosts in the field.

APPENDIX

RELATIONSHIP BETWEEN THE SEQUENTIAL JULIAN AND THE MONTHLY CALENDAR

Month-Day	Julian Date
January-1	1
February-1	32
March-1	60[1]
April-1	91
May-1	121
June-1	152
July-1	182
August-1	213
September-1	244
October-1	274
November-1	305
December-1	335

1 Add 1 to each of the following dates during a leap year.

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